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Plasma-mediated Release of Morphine from Synthesized Prodrugs

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Abstract: Two morphine prodrugs ('PDA' and 'PDB') were synthesized and the kinetics of esterase-mediated morphine release from these prodrugs were determined when incubated with plasma from different animal species. Morphine was rapidly released from PDA by all species' plasma with the maximum reached within 5-10 minutes; the released morphine was biologically active as determined by an in vitro cAMP assay. The morphine was released from PDB at a slower and species-dependent rate (mouse > rat > guinea pig > human). Morphine's release from PDB appeared to be mediated by carboxyl esterases as the release was inhibited by the carboxyl esterase inhibitor Benzil. PDA nor PDB induce cytotoxicity in the neuronal cell lines SK-NSH and SH-SY5Y. The carboxyl and amino functional moieties present on the linker portions of PDA and PDB, respectively, may facilitate their conjugation to nanoparticles to tailor morphine pharmacokinetics and specific targeting. These studies suggest the potential clinical utility of these prodrugs for morphine release at desired rates by administration of their mixture at selected ratios.

Morphine (MP) is an established standard in the treatment of acute and chronic pain.¹ However, morphine has a relatively short plasma half-life (1-3 h) associated with a correspondingly limited duration of analgesic effect.² Under certain circumstances, longer-acting morphine preparations may be desirable. Longer-acting, sustained-release oral morphine preparations are available, however, certain medical conditions may prevent oral administration of morphine e.g. inability to swallow, vomiting, bowel obstruction, etc.

Longer-acting, sustained-release morphine preparations for parenteral administration (e.g., intravenously, intramuscularly, subcutaneously) do not exist. Frequent parenteral injections or infusions may not be available, feasible, be uncomfortable or inconvenient. Therefore, morphine prodrugs with long half life which can release morphine in a slow, sustained fashion may be useful in the treatment of acute and chronic pain (e.g., from acute injuries sustained in remote, desolate places without healthcare facilities, cancer), avoiding the need for frequent parenteral morphine injections. Moreover, combinations of morphine prodrugs tailored for fast-, intermediate-, and long-acting release may be ideal for alleviating a range of acute and chronic pain conditions.

Because the free 3-OH group of morphine is a prerequisite for its analgesic activity,^{3, 4} different 3-substituted prodrugs have been synthesized and shown to have varying plasma release kinetics and biological activity.⁵⁻⁷ Our overall goal is to synthesize 3-substituted morphine prodrugs with linkers containing open functional groups which allow conjugation to nanoparticles, such as dendrimers,⁸ to change its pharmacokinetics and enable specific targeting. We report an abbreviated view of the synthesis of two morphine prodrugs and their morphine release kinetics in the presence of plasma from different animal species.

The structures of the synthesized morphine prodrugs, designated as prodrug A (PDA) and prodrug B (PDB), are shown in Figure 1. Their synthesis is summarized in the Supplementary Data section and in its Figure S-1.

-Figure 1-

Ultra Performance Liquid Chromatography (UPLC)⁹ (Waters Inc.) was utilized for measurements of morphine, PDA and PDB. UPLC has the capability to perform rapid (< 10 min) and reproducible chromatographic separations using small sample volumes compared to conventional High Performance Liquid Chromatography (HPLC) techniques,¹⁰ e.g. ~1-3 μ L for UPLC versus ~30-50 μ L for HPLC. The term “morphine” refers to the free morphine alkaloid base (Malinkrodt, etc.) unless otherwise stated.

Baseline UPLC profiles were obtained for phosphate buffered saline (PBS), morphine and PDA in esterase de-activated plasma. Plasma was precipitated by the addition of 2 volumes of 10% DMSO in acetonitrile (ANDM). This pretreated plasma was then spiked with morphine or PDA, and the supernatant was subjected to UPLC. ANDM allows both deproteinization as well as the release of protein-bound MP.¹¹

-Figure 2-

Baseline UPLC values of ANDM treated human plasma showed major peaks at 0.65, 0.89, 2.2, 2.4, 5.1, 5.4, 8.2 and 10.7 min. (Figure 2). PDA in ANDM treated human plasma eluted out at 4.3-4.4 min, while morphine appeared as dual peaks, with a major peak at 1.65-1.75 min., and a shoulder or a minor peak at 1.60-1.65 min. UPLC peaks of ANDM treated human plasma did not overlap the elution time peaks of morphine or PDA. When morphine and PDA were diluted in PBS and then subjected to similar UPLC analyses (data not shown), elution times for morphine and PDA were similar to those observed in ANDM treated human plasma, indicating that ANDM treated plasma components did not influence the elution profile of these two drugs.

To quantify the amount of morphine released, a standard curve was generated by injection of different amounts of morphine in PBS into the UPLC column and quantifying the areas under the peak curves (1.6 to 1.75 min.) for each concentration. The morphine peak area was linear from 10 nM to 3.5 mM morphine injected (Figure S-2), with an R value of 0.99.

-Figure 3-

The effect of plasma from different animal species on the kinetics of the release of morphine from PDA was determined. Most morphine was released within 5-10 minutes in the presence of plasma (Figure 3). Concurrent with the increase in the morphine peak, over >90% of the PDA peak (eluting at 4.3-4.4 min) disappeared at all incubation conditions. While PDA was stable in PBS under similar conditions for up to 24 hrs (data not shown), the rapid release of morphine was likely due to plasma components such as plasma esterases. A comparison of the effects of rat and guinea pig plasma and serum on PDA showed that they both induced the release of morphine with equal potency (data not shown), showing that neither the blood coagulation proteins nor the added anticoagulant in the plasma samples influence the hydrolytic process.

The released morphine was tested for biological activity by determining its known μ -opioid receptor mediated signal transduction property to decrease the cellular cAMP content.¹² We utilized the human neuronal cell line SK-NSH known to have cell surface μ -opioid receptors expressed.¹³ cAMP was quantified by a chemiluminescent assay using a cAMP standard curve (Figure S-3). Morphine released from PDA significantly inhibited forskolin-stimulated cAMP (forskolin is an activator of adenylate cyclase which increases the basal cellular cAMP) (Figure 4). PDA incubated in PBS under identical conditions failed to significantly inhibit cAMP production. These results show that the morphine released from the prodrug is biologically active.

-Figure 4-

Subsequently, the kinetics of morphine's release from prodrug B (PDB) by plasma from different species was tested. Figure 5 illustrates the UPLC profile obtained for the time-dependent release of morphine by rat plasma. The PDB eluted out at 4.4-4.6 min, while the released morphine did so at 1.6-1.8 min. With rat plasma, about 50% of the morphine released occurred in 30 min, reaching a maximum within 60 min. We also observed the concurrent, time-dependent increase of a second peak at 4.22-4.32 min, which probably represented the aromatic linker cleaved off from PDB (See Figure 1 and Figure S-4).

-Figure 5-

The rate of release of morphine from PDB by plasma of different species is shown in Figure 6. Among the different plasmas tested, the rate of hydrolysis was highest with mouse plasma, followed by rat > guinea pig > and human plasma. The higher hydrolytic activity in the rodents may be due to the presence of large amounts of serum carboxylesterases, as compared to insignificant levels observed in the human serum.¹⁴ In contrast to our observation, a previous study has shown that human plasma hydrolyzed morphine-3-propionate at a significantly higher rate than rat plasma,¹⁵ suggesting different ester substrates may have different specificity and esterase hydrolytic activity.

-Figure 6-

Butyryl choline esterase (BCE) and human serum albumin (HSA) have been suggested to play a role in ester hydrolysis.¹⁴ We also determined the effects of 25 mg/ml HSA and 0.5 Unit/ml purified human serum BCE, concentrations equivalent to 50% of their known levels in human serum.¹⁴ HSA released ~1% of morphine from PDB in 15 min., which reached a maximum of ~2% in 3-6 hrs. The BCE released ~2.5% morphine from PDB in 15 min, reaching to ~4% in 3-6 hrs. Under similar assay conditions, 50% human plasma released ~4% morphine in 15 min and ~20% in 6 hrs. These results show that PDB is a poor substrate for BCE and HSA, however, other carboxyl esterases¹⁶ or aryl esterase¹⁷ may participate in PDB's hydrolysis. These findings are similar to those measured for hydrolysis of the prodrug irinotecan by human plasma,¹⁸ but different from the known BCE hydrolysis of heroin which carries an acetate group at the 3-OH group of morphine.¹⁹

To verify the role of carboxyl esterase in mediating the hydrolysis of prodrugs A and B, we tested the effect of the carboxyl esterase inhibitor benzil on plasma-induced morphine release.^{16, 20} As shown in Figure 7, benzil inhibited morphine release from PDB in a dose-dependent fashion, suggesting the role of carboxyl esterase in PDB hydrolysis. However, under similar conditions of incubations, the rapid release of morphine from PDA was not inhibited by benzil (results not shown), suggesting possible high carboxyl esterase affinity for PDA vs. benzil, or the role of other esterases in PDA's hydrolysis.

-Figure 7-

Table 1 facilitates comparison of the levels of morphine that can be achieved by various species, based on their adult blood volumes assuming the administration of PDB doses in excess to the desired release.

-Table 1-

In order for the synthesized prodrugs to be useful for biological application, it is important that

they do not induce cytotoxicity at relevant concentrations. XTT assay²¹ of PDA and PDB showed that these prodrugs are not cytotoxic up to 1 μ M in the neuronal cell lines SK-NSH and SH-SY5Y (Figure 8).

-Figure 8-

In summary, we have demonstrated differential plasma hydrolytic activities for two novel morphine prodrugs carrying chemically functional 3-substituted groups. Future studies will focus on the pharmacokinetics of the conjugates administered individually or as a mixture. The prodrugs can be further utilized for conjugation to nanoparticles such as the neutralized and non-cytotoxic dendrimers,^{22, 23} which may allow improved drug solubility, payload, pharmacokinetics, and controlled release.

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KEY WORDS

Morphine, prodrugs, plasma, esterase, cytotoxicity

FIGURE CAPTIONS

Figure 1. Chemical structures of morphine prodrugs A and B.

Figure 2. UPLC profile of human plasma spiked with morphine (MP) or PDA. Human plasma was first mixed with ice-cold ANDM, then spiked with morphine (green), PDA (red), or PBS (black) followed by centrifugation. The protein-free supernatant from each of these separate sample mixtures was subjected to UPLC, using conditions described in the Supplementary Data.

Figure 3. The kinetics of the release of morphine from PDA by plasma from 4 species. 250 μ M of PDA was incubated with 50% plasma in PBS at 37°C, then aliquots were withdrawn at different time intervals, mixed with 2 volumes of ice-cold ANDM solution and centrifuged to remove the protein precipitate. The supernatant was subjected to UPLC analysis, and concentrations of the released morphine were calculated from standard curve generated for morphine.

Figure 4. The effect of vehicle- and plasma-incubated PDA on forskolin-stimulated cAMP in SK-NSH cells. 0.8 mM PDA was incubated with PBS or 50% human plasma (HP) for 24 hrs. SK-NSH cells plated in 24-well plates were changed to serum-free medium and incubated with the control- or plasma-incubated PDA solutions (equivalent to 5 μ M prodrug) for 15 min, followed by 15-min incubation with 5 μ M forskolin (FSK) or its vehicle. The cells were rinsed, lysed and the cAMP content was determined from a cAMP calibration curve (Figure 2S), using the “GraphPad Prism” software. * $p < 0.05$ vs. Control and ‘PDA + PBS’.

Figure 5. UPLC profile of the release of morphine from PDB by rat plasma, showing the time-dependent increase in morphine (A) and the corresponding decrease in PDB (B). The control plasma profile is indicated as ‘C’. The ‘spiked MP’ shown (solid black) is rat plasma spiked with morphine (150 μ M) following treatment with ANDM, under similar conditions. The unlabeled graphs (coinciding with the 60’) are those obtained for 180 and 360 min. Other details are as given for Figure 2.

Figure 6. The kinetics of release of morphine from PDB by plasma from different animal species, purified human serum BCE (Sigma B4186, 0.5 Units/ml) and human serum albumin (Sigma 8763, 25 mg/ml). A. Morphine release quantified from the peak area of morphine. B. The corresponding disappearance of PDB quantified from the peak area of PDB. Other experimental details are as given for Figure 2 and in the Supplementary Data

Figure 7. Dose-dependent inhibition of plasma-mediated release of MP from PDB, by the carboxyl esterase inhibitor benzil. PDB was incubated with rat plasma in the presence of different concentrations of benzil for 10’, 30’ or 60’, and the released morphine was quantified by UPLC analysis. Other conditions are as given for Figure 6. The data is given as the percent of maximal morphine release obtained for the control sample at 60’.

Figure 8. Lack of cytotoxicity of PDA and PDB in SK-NSH and SH-SY5Y cell lines. Cells cultured in 96-well plates were incubated with different concentrations of pro-drugs for 3 days and the cell cytotoxicity was quantified by XTT assay.

Plasma/Enzyme Source	MP Release Rate, nmoles/min/ml	MP Release Rate, μg/min/ml	Adult Blood Vol, ml	Projected Release, mg/h/ Adult
Mouse	20.9	5.97	1.5	0.54
Rat	4.2	1.19	25	1.8
Guinea Pig	2.9	0.83	25	1.2
Human	1.1	0.31	4000	74.5
BCE	0.8	0.23		
HSA	0.3	0.10		

Table 1. Comparison of the rate of hydrolysis of PDB by different plasmas, human BCE and HSA. The data is derived from the 0 to 15 minute time points at which time the rates were linear for all samples (ref: Figure 6). The reaction mixture contained an initial amount of 37.5 nmoles of PDB and 150 μ l plasma/enzyme samples in a total volume of 300 μ l. The rates given are per ml of plasma, or enzyme samples at known levels in human plasma.























